DECLARATION

I, Makoto AIHARA, Patent Attorney, of SIKs & Co., 8th Floor, Kyobashi-Nisshoku Building, 8-7, Kyobashi 1-chome, Chuo-ku, Tokyo 104-0031 JAPAN hereby declare that I am the translator of the certified official copy of the documents in respect of an application for a patent filed in Japan on September 20, 2002 under Patent Application No.274266/2002 and that the following is a true and correct translation to the best of my knowledge and belief.

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[List of Attached Documents]

[Document's Name]	Specification	1
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[Name of Document] SPECIFICATION

[Title of Invention] FLUORESCENT PROTEIN

[Claims]

[Claim 1] A fluorescent protein derived from *Trachyphyllia geoffroyi*, which has the following properties:

(1) the color is changed from green to red by irradiation of ultraviolet ray; the excitation maximum wavelength is 508 nm (green) and 572 nm (red); and the fluorescence maximum wavelength is 518 nm (green) and 581 nm (red);

(2) the molar absorption coefficient (green) at 508 nm is 98800 $M^{-1}cm^{-1}$; and the molar absorption coefficient (red) at 572 nm is 60400 $M^{-1}cm^{-1}$;

(3) the quantum yield is 0.80 (green) and 0.33 (red); and

(4) pKa regarding the pH sensitivity of the green and red are both 5.7.

[Claim 2] A fluorescent protein having either one of the following amino acid sequences:

(a) an amino acid sequence shown in SEQ ID NO: 1; or

(b) an amino acid sequence comprising a deletion, substitution and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and having fluorescent properties.

[Claim 3] A DNA of either one of the following:

(a) DNA which encodes the amino acid sequence shown in SEQ ID NO: 1; or

(b) DNA which encodes an amino acid sequence comprising a deletion, substitution and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and encodes a fluorescent protein:

(c) DNA having a nucleotide sequence shown in SEQ ID NO: 2; or

(d) DNA having a nucleotide sequence comprising a deletion, substitution and/or addition of one or several nucleotides with respect of the nucleotide sequence shown in SEQ ID NO: 2, and encoding a fluorescent protein.

[Claim 4] A recombinant vector having the DNA of claim 3.

[Claim 5] A transformant having any of the DNA of claim 3 or the recombinant

vector of claim 4.

[Claim 6] A fusion fluorescent protein consisting of the fluorescent protein of claim 1 or 2 and another protein.

[Claim 7] The fusion fluorescent protein of claim 6 wherein said another protein is one that localizes in the cell,.

[Claim 8] The fusion fluorescent protein of claim 6 or 7 wherein said another protein is one specific to an intracellular organella.

[Claim 9] A method for analyzing the localization or dynamics of a protein in cells, characterized in that the fusion protein of any of claims 6 to 8 is allowed to be expressed in cells.

[Claim 10] A fluorescent reagent kit which comprises the fluorescent protein of claim 1 or 2, the DNA of claim 3, the recombinant vector of claim 4, the transformant of claim 5, or the fusion fluorescent protein of any of claims 6 to8.

[Detailed Description of the Invention]

[0001]

[Technical Field of the Invention]

The present invention relates to a novel fluorescent protein. More specifically, the present invention relates to a novel fluorescent protein derived from *Trachyphyllia geoffroyi* and the use thereof.

[0002]

[Prior Art]

Green fluorescent protein (GFP) derived from *Aequorea victoria*, a jellyfish, has many purposes in biological systems. Recently, various GFP mutants have been produced based on the random mutagenesis and semi-rational mutagenesis, wherein a color is changed, a folding property is improved, luminance is enhanced, or pH sensitivity is modified. Fluorescent proteins such as GFP are fused with other proteins by gene recombinant technique, and monitoring of the expression and transportation of the fusion proteins is carried out.

[0003]

One of the most commonly used types of GFP mutant is Yellow fluorescent protein (YFP). Among Aequorea-derived GFP mutants, YFP exhibits the fluorescence with the longest wavelength. The values ε and Φ of the majority of YEPs are 60,000 to 100,000 $M^{-1}cm^{-1}$ and 0.6 to 0.8, respectively (Tsien, R. Y. (1998). Ann. Rev. Biochem. 67, 509-544). These values are comparable to those of the general fluorescent group (fluorescein, rhodamine, etc.). Accordingly, improvement of the absolute luminance of YFP is nearly approaching its limit.

[0004]

In addition, cyan fluorescent protein (CFP) is another example of the GFP mutant. Of this type of protein, ECFP (enhanced cyan fluorescent protein) has been known. Moreover, red fluorescent protein (RFP) has been isolated from sea anemone (*Discoma sp.*). Of this type of protein, DasRed has been known. Thus, 4 types of fluorescent proteins, that are, green fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, and red fluorescent protein, have successively been developed. The range of the spectrum has significantly been expanded.

[0005]

[Object to be Solved by the Invention]

It is an object of the present invention to provide a fluorescent protein having a novel primary structure, which is derived from Cnidaria, and particularly from *Trachyphyllia geoffroyi* belonging to Scleractinia.

[0006]

[Means for Solving the Object]

The present inventors have conducted intensive studies directed towards achieving the aforementioned object. The present inventors have performed expression cloning by using cDNA library derived from *Trachyphyllia geoffroyi,* and have succeeded in cloning genes encoding novel fluorescent protein. The present inventors have examined the fluorescent properties of the obtained fluorescent proteins, and as a result, they have found that these fluorescent proteins have particular fluorescent properties. The present invention has been completed based on these findings.

[0007]

Thus, the present invention provides a fluorescent protein derived from *Trachyphyllia geoffroyi*, which has the following properties:

(1) the color is changed from green to red by irradiation of ultraviolet ray; the excitation maximum wavelength is 508 nm (green) and 572 nm (red); and the fluorescence maximum wavelength is 518 nm (green) and 581 nm (red);

(2) the molar absorption coefficient (green) at 508 nm is 98800 $M^{-1}cm^{-1}$; and the molar absorption coefficient (red) at 572 nm is 60400 $M^{-1}cm^{-1}$;

(3) the quantum yield is 0.80 (green) and 0.33 (red); and

(4) pKa regarding the pH sensitivity of the green and red are both 5.7.

[0008]

In another aspect of the present invention, there is provided a fluorescent protein having either one of the following amino acid sequences:

(a) an amino acid sequence shown in SEQ ID NO: 1; or

(b) an amino acid sequence comprising a deletion, substitution and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and having fluorescent properties.

[0009]

In further another aspect of the present invention, there is provided a DNA of either one of the following:

(a) DNA which encodes the amino acid sequence shown in SEQ ID NO: 1; or

(b) DNA which encodes an amino acid sequence comprising a deletion, substitution and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and encodes a fluorescent protein:

(c) DNA having a nucleotide sequence shown in SEQ ID NO: 2; or

(d) DNA having a nucleotide sequence comprising a deletion, substitution and/or addition of one or several nucleotides with respect of the nucleotide sequence shown in SEQ ID NO: 2, and encoding a fluorescent protein.

[0010]

In further another aspect of the present invention, there is provided a recombinant vector having any of the DNA of the present invention.

In further another aspect of the present invention, there is provided a transformant having the DNA or recombinant vector of the present invention.

In further another aspect of the present invention, there is provided a fusion fluorescent protein consisting of the fluorescent protein of the present invention and another protein. Preferably, said another protein is one that localizes in the cell, and more preferably one specific to an intracellular organella.

[0011]

In further another aspect of the present invention, there is provided a method for analyzing the localization or dynamics of a protein in cells, characterized in that the fusion protein of the present invention is allowed to be expressed in cells.

In further another aspect of the present invention, there is provided a fluorescent reagent kit which comprises the fluorescent proteins, DNAs, recombinant vector, transformant or fusion protein of the present invention.

[0012]

[Embodiment for Carrying out the Invention]

The embodiments of the present invention will be described in detail below.

(1) Fluorescent protein of the present invention

The fluorescent protein of the present invention is characterized in that it is derived from *Trachyphyllia geoffroyi* and has the following properties:

(1) the color is changed from green to red by irradiation of ultraviolet ray; the excitation maximum wavelength is 508 nm (green) and 572 nm (red); and the fluorescence maximum wavelength is 518 nm (green) and 581 nm (red);

(2) the molar absorption coefficient (green) at 508 nm is 98800 $M^{-1}cm^{-1}$; and the molar absorption coefficient (red) at 572 nm is 60400 $M^{-1}cm^{-1}$;

(3) the quantum yield is 0.80 (green) and 0.33 (red); and

(4) pKa regarding the pH sensitivity of the green and red are both 5.7.

[0013]

Trachyphyllia geoffroyi is one type of cnidarian sea anemone, and it is characterized in that it emits extremely colorful fluorescence. *Trachyphyllia geoffroyi* mainly ranges over the area below the midland of Honshu Island, Japan. This sea anemone lives in the sludge in the gulf. At night, it extends its tentacles to capture plankton and the like. In terms of a color variation, green, brown, and red examples are found.

[0014]

As shown in the examples later, the color of the fluorescent protein of the present invention is changed from green to red by irradiation of ultraviolet ray; the excitation maximum wavelength is 508 nm (green) and 572 nm (red); and the fluorescence maximum wavelength is 518 nm (green) and 581 nm (red). The molar absorption coefficient (green) at 508 nm is 98800 $M^{-1}cm^{-1}$; and the molar absorption coefficient (red) at 572 nm is 60400 $M^{-1}cm^{-1}$;

[0015]

The fluorescent protein of the present invention is characterized in that its color changes due to ultraviolet rays. Thus, optical marking can be carried out on specific cells or organs thereof.

[0016]

The examples of the fluorescent protein of the present invention include a fluorescent protein having either one of the following amino acid sequences:

(a) an amino acid sequence shown in SEQ ID NO: 1; or

(b) an amino acid sequence comprising a deletion, substitution and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and having fluorescent properties.

[0017]

The scope of "one or several" in the phrase "an amino acid sequence comprising a deletion, substitution and/or addition of one or several amino acids" is not particularly limited in the present specification. For example, it means 1 to 20, preferably 1 to 10, more preferably 1 to 7, further preferably 1 to 5, and particularly preferably 1 to 3.

[0018]

The term "having fluorescent properties" covers all of the cases where any fluorescence is given. Various properties such as fluorescence intensity, excitation wavelength, fluorescence wavelength or pH sensitivity, may be changed or may remain unchanged, as compared with those of the protein having an amino acid sequence of SEQ ID NO.1.

[0019]

The method of obtaining the fluorescent protein of the present invention is not particularly limited. The protein may be either a protein synthesized by chemosynthesis, or recombinant protein produced by a gene recombination technique.

Where a recombinant protein is produced, it is necessary to obtain DNA encoding the protein. Appropriate primers are designed by using information regarding the amino acid sequence shown in SEQ ID NO: 1 of the sequence listing of the present specification and the nucleotide sequence shown in SEQ ID NO: 2 thereof. Using these primers, PCR is carried out by using cDNA clones of the above-described various types of known fluorescent proteins as a template, so that DNA encoding the fluorescent protein of the present invention can be obtained. Where a partial fragment of DNA encoding the fluorescent protein of the present invention are obtained by the above-described PCR, the produced DNA fragments are ligated to one another by a gene recombination technique, so that DNA encoding the desired fluorescent protein can be obtained. The fluorescent protein of the present invention can be produced by introducing this DNA into an appropriate expression system. Expression in an expression system will be described later in the present specification.

[0020]

(3) DNA of the present invention

According to the present invention, genes encoding the fluorescent protein of the present invention are provided.

Specific examples of DNA encoding the first fluorescent protein of the present invention may include either one of the following DNAs:

(a) DNA which encodes the amino acid sequence shown in SEQ ID NO: 1; or

(b) DNA which encodes an amino acid sequence comprising a deletion, substitution and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and encodes a fluorescent protein:

(c) DNA having a nucleotide sequence shown in SEQ ID NO: 2; or

(d) DNA having a nucleotide sequence comprising a deletion, substitution and/or addition of one or several nucleotides with respect of the nucleotide sequence shown in SEQ ID NO: 2, and encoding a fluorescent protein.

[0021]

The DNA of the present invention can be synthesized by, for example, the phosphoamidite method, or it can also be produced by polymerase chain reaction (PCR) using specific primers. The DNA of the present invention is produced by the method described above in the specification.

[0022]

A method of introducing a desired mutation into a certain nucleic acid sequence is known to a person skilled in the art. For example, known techniques such as a site-directed mutagenesis, PCR using degenerated oligonucleotides, or the exposure of cells containing nucleic acid to mutagens or radioactive rays, are appropriately used, so as to construct DNA having a mutation. Such known techniques are described in, for example, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY., 1989; and Current Protocols in Molecular Biology, Supplements 1 to 38, John Wiley & Sons (1987-1997).

[0023]

(3) Recombinant vector of the present invention

The DNA of the present invention can be inserted into a suitable vector and used. The type of a vector used in the present invention is not particularly limited. For example, it may be either a vector that can autonomously replicate (e.g., a plasmid, etc.), or vector that is incorporated into the genomes of host cells when it is introduced into the host cells and is then replicated together with the chromosome into which it is

incorporated.

[0024]

The vector used in the present invention is preferably an expression vector. In an expression vector, elements necessary for transcription (e.g., a promoter, etc.) are functionally ligated to the DNA of the present invention. The promoter is a DNA sequence which shows a transcriptional activity in host cells, and it is appropriately selected depending on the type of host cells.

[0025]

Examples of a promoter which can operate in bacterial cells may include a *Bacillus stearothermophilus* maltogenic amylase gene promoter, a *Bacillus licheniformis* alpha-amylase gene promoter, a *Bacillus amyloliquefaciens* BAN amylase gene promoter, a *Bacillus subtilis* alkaline protease gene promoter, a *Bacillus pumilus* xylosidase gene promoter, P_R and P_L promoters of phage rhamda, and lac, trp and tac promoters of *Escherichia coli*.

[0026]

Examples of a promoter which can operate in mammalian cells may include an SV40 promoter, an MT-1 (metallothionein gene) promoter, and an adenovirus-2 major late promoter. Examples of a promoter which can operate in insect cells may include a polyhedrin promoter, a P10 promoter, an *Autographa californica* polyhedrosis basic protein promoter, a baculovirus immediate-early gene 1 promoter, and a baculovirus 39K delayed-early gene promoter. Examples of a promoter which can be operate in yeast host cells may include promoters derived from yeast glycolytic genes, an alcohol dehydrogenase gene promoter, a TPI1 promoter, and an ADH2-4c promoter.

Examples of a promoter which can operate in filamentous cells may include an ADH3 promoter and a tpiA promoter.

[0027]

In addition, an appropriate terminator such as a human growth hormone terminator, or a TPI1 terminator or ADH3 terminator for fungal cells, may be functionally bound to the DNA of the present invention, as necessary. The recombinant

vector of the present invention may further have elements such as a polyadenylation signal (e.g., one derived from SV40 or the adenovirus 5E1b region), a transcription enhancer sequence (e.g., an SV40 enhancer), or a translation enhancer sequence (e.g., one encoding the adenovirus VA RNA).

The recombinant vector of the present invention may further comprise a DNA sequence which enables the replication of the recombinant vector in host cells. SV40 replication origin is an example of such a sequence (when the host cells are mammalian cells).

[0028]

The recombinant vector of the present invention may further comprise a selective marker. Examples of such a selective marker may include genes, complements of which are absent from host cells, such as a dihydrofolate reductase (DHFR) gene or a *Shizosaccharomyces pombe* TPI gene, and drug resistant genes such as ampicillin, kanamycin, tetracycline, chloramphenicol, neomycin or hygromycin-resistant genes.

A method for ligating the DNA of the present invention, a promoter and, as desired, a terminator and/or a secretory signal sequence to one another and inserting these items into a suitable vector is known to a person skilled in the art.

[0029]

(4) Transformant of the present invention

A transformant can be produced by introducing the DNA or recombinant vector of the present invention into a suitable host.

Any cell can be used as a host cell into which the DNA or recombinant vector of the present invention is introduced, as long as the DNA construct of the present invention can be expressed therein. Examples of such a cell may include bacteria, yeasts, fungal cells, and higher eukaryotic cells.

[0030]

Examples of bacteria may include Gram-positive bacteria such as Bacillus or Streptomyces, and Gram-negative bacteria such as *Escherichia coli*. These bacteria may be transformed by the protoplast method or other known methods, using competent

cells.

Examples of mammalian cells may include HEK 293 cells, HeLa cells, COS cells, BHK cells, CHL cells, and CHO cells. A method of transforming mammalian cells and expressing the introduced DNA sequence in the cells is also known. Examples of such a method may include the electroporation, the calcium phosphate method, and the lipofection method.

[0031]

Examples of yeast cells may include those belonging to Saccharomyces or Shizosaccharomyces. Examples of such cells may include *Saccharomyces cerevisiae* and *Saccharomyces kluyveri*. Examples of a method of introducing a recombinant vector into yeast host cells may include the electroporation, the spheroplast method, and the lithium acetate method.

[0032]

Examples of other fungal cells may include those belonging to *Filamentous fungi* such as Aspergillus, Neurospora, Fusarium or Trichoderma. Where *Filamentous fungi* are used as host cells, transformation can be carried out by incorporating DNA constructs into host chromosomes, so as to obtain recombinant host cells. Incorporation of DNA constructs into the host chromosomes is carried out by known methods, and such known methods may include homologous recombination and heterologous recombination.

[0033]

Where insect cells are used as host cells, both a vector into which a recombinant gene is introduced and a baculovirus are co-introduced into insect cells, and a recombinant virus is obtained in the culture supernatant of the insect cells. Thereafter, insect cells are infected with the recombinant virus, so as to allow the cells to express proteins (described in, for example, Baculovirus Expression Vectors, A Laboratory Manual; and Current Protocols in Molecular Biology, Bio/Technology, 6, 47 (1988)).

[0034]

The *Autographa californica* nuclear polyhedrosis virus, which is a virus

infecting to insects belonging to *Barathra brassicae*, can be used as baculovirus.

Examples of insect cells used herein may include Sf9 and Sf21, which are *Spodoptera frugiperda* ovarian cells [Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman & Company, New York, (1992)], and HiFive (manufactured by Invitrogen), which are *Trichoplusia ni* ovarian cells.

Examples of the method of co-introducing both a vector into which a recombinant gene has been introduced and the above baculovirus into insect cells to prepare a recombinant virus may include the calcium phosphate method and the lipofection method.

[0035]

The above transformant is cultured in an appropriate nutritive medium under conditions enabling the introduced DNA construct to be expressed. In order to isolate and purify the protein of the present invention from the culture product of the transformant, common methods of isolating and purifying proteins may be used.

For example, where the protein of the present invention is expressed in a state dissolved in cells, after completion of the culture, cells are recovered by centrifugal separation, and the recovered cells are suspended in a water type buffer. Thereafter, the cells are disintegrated using an ultrasonic disintegrator or the like, so as to obtain a cell-free extract. A supernatant is obtained by centrifuging the cell-free extract, and then, a purified sample can be obtained from the supernatant by applying, singly or in combination, the following ordinary protein isolation and purification methods: the solvent extraction, the salting-out method using ammonium sulfate or the like, the desalting method, the precipitation method using an organic solvent, the anion exchange chromatography using resins such as diethylaminoethyl (DEAE) sepharose, the cation exchange chromatography using resins such as S-Sepharose FF (manufactured by Pharmacia), the hydrophobic chromatography using resins such as butyl sepharose or phenyl sepharose, the gel filtration method using a molecular sieve, the affinity chromatography, the chromatofocusing method, and the electrophoresis such as isoelectric focusing.

[0036]

(5) Use of the fluorescent protein of the present invention and a fusion fluorescent protein comprising the same

The fluorescent protein of the present invention can be fused with another protein, so as to construct a fusion fluorescent protein.

A method of obtaining the fusion fluorescent protein of the present invention is not particularly limited. It may be either a protein synthesized by chemosynthesis, or recombinant protein produced by a gene recombination technique.

Where a recombinant protein is produced, it is necessary to obtain DNA encoding the protein. Appropriate primers are designed using the information regarding the amino acid sequence shown in SEQ ID NO: 1 of the sequence listing of the present specification and the nucleotide sequence shown in SEQ ID NO: 2 thereof. Using these primers, PCR is carried out using a DNA fragment containing the gene of the fluorescent protein of the present invention as a template, so as to produce DNA fragments necessary for construction of the DNA encoding the fluorescent protein of the present invention. Moreover, DNA fragment encoding a protein to be fused is also obtained in the same above manner.

[0037]

Subsequently, the thus obtained DNA fragments are ligated to one another by a gene recombination technique, so that DNA encoding the desired fusion fluorescent protein can be obtained. This DNA is then introduced into an appropriate expression system, so that the fusion fluorescent protein of the present invention can be produced.

[0038]

The fluorescent protein of the present invention has an extremely high utility value as a marker. This is to say, the fluorescent protein of the present invention is purified as a fusion protein with an amino acid sequence to be tested, and the fusion protein is introduced into cells by methods such as the microinjection. By observing the distribution of the fusion protein over time, targeting activity of the amino acid sequence to be tested can be detected in the cells.

[0039]

The type of another protein (an amino acid sequence to be tested) with which the fluorescent protein of the present invention is fused is not particularly limited. Preferred examples may include proteins localizing in cells, proteins specific for intracellular organelles, and targeting signals (e.g., a nuclear transport signal, a mitochondrial presequence, etc.). In addition, the fluorescent protein of the present invention can be expressed in cells and used, as well as being introduced into cells by the microinjection or the like. In this case, a vector into which the DNA encoding the fluorescent protein of the present invention is inserted in such a way that it can be expressed, is introduced into host cells.

[0040]

Moreover, the fluorescent protein of the present invention can also be used as a reporter protein to determine promoter activity. This is to say, a vector is constructed such that DNA encoding the fluorescent protein of the present invention is located downstream of a promoter to be tested, and the vector is then introduced into host cells. By detecting the fluorescence of the fluorescent protein of the present invention which is emitted from the cells, the activity of the promoter to be tested can be determined. The type of a promoter to be tested is not particularly limited, as long as it operates in host cells.

[0041]

A vector used to detect the targeting activity of the above amino acid sequence to be tested or to determine promoter activity is not particularly limited. Examples of a vector preferably used for animal cells may include pNEO (P. Southern, and P. Berg (1982) J. Mol. Appl. Genet. 1: 327), pCAGGS (H. Niwa, K. Yamamura, and J. Miyazaki, Gene 108, 193-200 (1991)), pRc/CMV (manufactured by Invitrogen), and pCDM8 (manufactured by Invitrogen). Examples of a vector preferably used for yeasts may include pRS303, pRS304, pRS305, pRS306, pRS313, pRS314, pRS315, pRS316 (R. S. Sikorski and P. Hieter (1989) Genetics 122: 19-27), pRS423, pRS424, pRS425, pRS426 (T. W. Christianson, R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter (1992) Gene

110: 119-122).

[0042]

In addition, the type of cells used herein is also not particularly limited. Various types of animal cells such as L cells, BalbC-3T3 cells, NIH3T3 cells, CHO (Chinese hamster ovary) cells, HeLa cells or NRK (normal rat kidney) cells, yeast cells such as *Saccharomyces cerevisiae*, *Escherichia coli* cells, or the like can be used. Vector can be introduced into host cells by common methods such as the calcium phosphate method or the electroporation.

[0043]

The above obtained fusion fluorescent protein of the present invention wherein the fluorescent protein of the present invention is fused with another protein (referred to as a protein X) is allowed to be expressed in cells. By monitoring a fluorescence emitted, it becomes possible to analyze the localization or dynamics of the protein X in cells. That is, cells transformed or transfected with DNA encoding the fusion fluorescent protein of the present invention are observed with a fluorescence microscope, so that the localization and dynamics of the protein X in the cells can be visualized and thus analyzed.

[0044]

For example, by using a protein specific for an intracellular organella as a protein X, the distribution and movement of a nucleus, a mitochondria, an endoplasmic reticulum, a Golgi body, a secretory vesicle, a peroxisome, etc., can be observed.

Moreover, for example, axis cylinders or dendrites of the nerve cells show an extremely complicated change in strikes in an individual who is under development. Accordingly, fluorescent labeling of these sites enables a dynamic analysis.

[0045]

The fluorescence of the fluorescent protein of the present invention can be detected with a viable cell. Such detection can be carried out using, for example, a fluorescence microscope (Axiophoto Filter Set 09 manufactured by Carl Zeiss) or an image analyzer (Digital Image Analyzer manufactured by ATTO).

The type of a microscope can be appropriately selected depending on purposes. Where frequent observation such as pursuit of a change over time is carried out, an ordinary incident-light fluorescence microscope is preferable. Where observation is carried out while resolution is emphasized, for example, in the case of searching localization in cells specifically, a confocal laser scanning microscope is preferable. In terms of maintenance of the physiological state of cells and prevention from contamination, an inverted microscope is preferable as a microscope system. When an erecting microscope with a high-powered lens is used, a water immersion lens can be used.

[0046]

A filter set can be appropriately selected depending on the fluorescence wavelength of a fluorescent protein. In the case of the fluorescent protein of the present invention, when the green having the excitation maximum wavelength of 508 nm and the fluorescence maximum wavelength of 518 nm is detected, a filter having an excitation light between approximately 490 and 510 nm and a fluorescence between approximately 510 and 530 nm can be preferably used. Also, when the red having the excitation maximum wavelength of 572 nm and the fluorescence maximum wavelength of 581 nm is detected, a filter having an excitation light between approximately 560 and 575 nm and a fluorescence between approximately 575 and 590 nm can be preferably used.

[0047]

When viable cells are observed over time using a fluorescence microscope, a high sensitive cooled CCD camera is used, since photography is carried out in a short time. In the case of the cooled CCD camera, CCD is cooled to decrease thermal noise, so that a weak fluorescence image can be clearly photographed by exposure in a short time.

[0048]

(6) Kit of the present invention

The present invention provides a kit for analyzing the localization of intracellular components and/or analyzing physiologically active substances, which is

characterized in that it comprises at least one selected from the fluorescent protein, the fusion fluorescent protein, the DNA, the recombinant vector, or the transformant, which are described in the present specification. The kit of the present invention can be produced from commonly used materials that are known per se, by using common methods.

Reagents such as the fluorescent protein or the DNA are dissolved in an appropriate solvent, so that the reagents can be prepared in a form suitable for conservation. Water, ethanol, various types of buffer solution, etc. can be used as such a solvent.

The present invention will be further described in the following examples. However, the present invention is not limited by these examples.

[0049]

[Examples]

Example 1: Isolation of novel fluorescent protein gene (Kaede) from coral

A fluorescent protein gene was isolated from *Trachyphyllia geoffroyi* which emits fluorescence with rich colors by the following procedures.

(1) Extraction of total RNA

Total RNA was extracted by acidic guanidium/phenol/chloroform method.

Frozen *Trachyphyllia geoffroyi* was crushed in a denaturation solution by using a Multi-Beads Shocker (Yasui Kikai), and then phenol/chloroform was added thereto, followed by centrifugation to separate RNA from protein and DNA. A water phase containing RNA was added to isopropanol, and the mixture was centrifuged, so as to obtain total RNA as a precipitate.

[0050]

(2) Purification of RNA

Using Oligotex-dT30 (manufactured by Roche), mRNA was separated from the total RNA.

Oligotex-dT30<super> was added to the total RNA, and the mixture was then heated, so as to destroy the secondary structure of the RNA. Thereafter, the RNA was

bound to Oligotex-dT at 37°C. After washing, the resultant product was heated and centrifuged, so as to obtain a supernatant eluted from the mRNA. Oligotex-dt was eliminated from the supernatant, and then, mRNA was allowed be precipitated with ethanol and NaCl. The mRNA was then dissolved in water.

[0051]

(3) Preparation of cDNA

A cDNA fragment was prepared using TimeSaver and Directional Cloning Toolbox (both of which were manufactured by Amersham Pharmacia).

The mRNA was heated to destroy the secondary structure thereof. Thereafter, the mRNA, DTT, and a *Not*I-dT primer were added to First-Strand Reaction Mix, so as to synthesize a first strand. This was then added to Second-Strand Reaction Mix, so as to synthesize a second strand. The synthesized second strand was purified with a span column attached with the kit. *Eco*RI adaptors were added to both termini of the purified double-stranded cDNA, and only the 3′-side thereof was cleaved with *Not*I. It was purified again with the span column, so as to obtain a cDNA fragment (*Eco*RI- *Not*I).

[0052]

(4) Expression Cloning

An *Eco*RI-*Not*I site was made in pRSETB (manufactured by Invitrogen), and the prepared cDNA was inserted into the site. Thereafter, the thus prepared vector was introduced into *Escherichia coli* JM109 DM3, followed by culture on an LA plate. Since a protein is synthesized in this strain, colonies that emit fluorescence when UV is applied were isolated.

As a result, 2 colonies emitting fluorescence were obtained from approximately 130,000 colonies. The nucleotide sequence thereof was determined with a DNA sequencer. This clone was named as Kaede. The amino acid sequence of Kaede is shown in SEQ ID NO: 1 of the sequence listing, and the nucleotide sequence of Kaede is shown in SEQ ID NO: 2.

[0053]

(5) Analysis of fluorescence properties

(a) Expression and purification of protein

A *Bam*HI site was added to the N-terminus of the obtained full-length cDNA, and an *Eco*RI site was added to the C-terminus thereof. Thereafter, it was subcloned in frame into pRSETB (manufactured by Invitrogen), and it was then expressed in *Escherichia coli* JM109 DE3. The expressed protein was purified with Ni-Agarose gel (manufactured by QIAGEN), utilizing an His-tag at the N-terminus thereof.

[0054]

(b) Absorption spectrum and molar absorption coefficient, and fluorescence spectrum and quantum yield

When this fluorescent protein is irradiated with UV, the absorption and fluorescence spectra thereof shift to long wavelengths, from green to red. Figure 1 shows the absorption spectrum of a purified protein before and after UV irradiation (the solid line represents the absorption spectrum before UV irradiation, and the dotted line represents the absorption spectrum after UV irradiation). The molar absorption coefficient was obtained from the concentration of the protein and the absorbance at absorption maximum (Table 1).

The fluorescence spectrum was measured by exciting the protein at 480 nm before and after UV irradiation (Figure 2), and the quantum yield was calculated by comparison with Fluorescein (manufactured by Molecular Probes) (Table 1).

The fluorescence properties of Kaede are shown in the following Table 1.

[0055]

[Table 1]

Fluorescence properties of Kaede

Excitation maximum (nm)	Fluorescence maximum (nm)	Molar absorption coefficient (M^{-1}/cm^{-1})	Quantum yield	pH sensitivity (pKa)	Number of amino acids
Green: 508nm Red: 572nm	Green: 518 nm Red: 581nm	Green: 98,800 (508 nm) Red: 60,400 (572 nm)	Green: 0.80 Red: 0.33	Green: 5.7 Red: 5.7	225

[0056]

(c) Properties regarding pH sensitivity

The absorption spectra of green and red were measured in a buffer each having pH 4 to 11. In both cases of green and red, the absorption level gradually decreases on reaching pH 9. pKa calculated from a change in the absorption maximum is shown in the above Table 1.

[0057]

Example 2: Introduction of novel fluorescent protein gene into mammalian cells

The Kaede gene was introduced into HeLa cells, using LIPOFECTIN Reagent (Gibco). Figure 3 shows the results obtained by exciting the cells at 470 nm and measuring them with the fluorescence at 510 nm. Fluorescence can be confirmed approximately 9 hours after the introduction. Fluorescence shifts to a long wavelength even in mammalian cells, when the cells are irradiated with UV.

[0058]

[Effect of the Invention]

The present invention provides a fluorescent protein having a novel primary structure derived from *Trachyphyllia geoffroyi*. The fluorescent protein of the present invention is characterized in that its color changes from green to red by ultraviolet rays. It is possible to carry out optical marking on specific cells or organs by light.

[0059]

[SEQUENCE LISTING]

SEQUENCE LISTING

<110> RIKEN

<120> Fluorescent proteins

<130> A21618A

<160> 2

[0060]

<210> 1

<211> 225

<212> PRT

<213> Trachyphyllia geoffroyi

<400> 1

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20 25 30

Pro Phe Glu Gly Lys Gln Ser Met Asp Leu Val Val Lys Glu Gly Ala
35 40 45

Pro Leu Pro Phe Ala Tyr Asp Ile Leu Thr Thr Ala Phe His Tyr Gly
50 55 60

Asn Arg Val Phe Ala Lys Tyr Pro Asp His Ile Pro Asp Tyr Phe Lys
65 70 75 80

Gln Ser Phe Pro Lys Gly Phe Ser Trp Glu Arg Ser Leu Met Phe Glu
85 90 95

Asp Gly Gly Val Cys Ile Ala Thr Asn Asp Ile Thr Leu Lys Gly Asp
100 105 110

Thr Phe Phe Asn Lys Val Arg Phe Asp Gly Val Asn Phe Pro Pro Asn
115 120 125

Gly Pro Val Met Gln Lys Lys Thr Leu Lys Trp Glu Ala Ser Thr Glu
130 135 140

Lys Met Tyr Leu Arg Asp Gly Val Leu Thr Gly Asp Ile Thr Met Ala
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Leu Leu Leu Lys Gly Asp Val His Tyr Arg Cys Asp Phe Arg Thr Thr
165 170 175
Tyr Lys Ser Arg Gln Glu Gly Val Lys Leu Pro Gly Tyr His Phe Val
180 185 190
Asp His Cys Ile Ser Ile Leu Arg His Asp Lys Asp Tyr Asn Glu Val
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Lys
225

[0061]

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<211> 678

<212> DNA

<213> Trachyphyllia geoffroyi

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Asn Val Asn Gly His Gln Phe Val Ile Glu Gly Asp Gly Lys Gly His
20 25 30
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Pro Phe Glu Gly Lys Gln Ser Met Asp Leu Val Val Lys Glu Gly Ala
35 40 45
cct ctc cct ttt gcc tac gat atc ttg aca aca gca ttc cat tat ggt 192
Pro Leu Pro Phe Ala Tyr Asp Ile Leu Thr Thr Ala Phe His Tyr Gly

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     50                  55                  60
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 65                  70                  75                  80
cag tcg ttt ccc aaa ggg ttt tct tgg gag cga agc ctg atg ttc gag   288
Gln Ser Phe Pro Lys Gly Phe Ser Trp Glu Arg Ser Leu Met Phe Glu
                 85                  90                  95
gac ggg ggc gtt tgc atc gct aca aat gac ata aca ctg aaa gga gac   336
Asp Gly Gly Val Cys Ile Ala Thr Asn Asp Ile Thr Leu Lys Gly Asp
            100                 105                 110
act ttt ttt aac aaa gtt cga ttt gat ggc gta aac ttt ccc cca aat   384
Thr Phe Phe Asn Lys Val Arg Phe Asp Gly Val Asn Phe Pro Pro Asn
        115                 120                 125
ggt cct gtt atg cag aag aag act ctg aaa tgg gag gca tcc act gag   432
Gly Pro Val Met Gln Lys Lys Thr Leu Lys Trp Glu Ala Ser Thr Glu
    130                 135                 140
aaa atg tat ttg cgt gat gga gtg ttg acg ggc gat att acc atg gct   480
Lys Met Tyr Leu Arg Asp Gly Val Leu Thr Gly Asp Ile Thr Met Ala
145                 150                 155                 160
ctg ctg ctt aaa gga gat gtc cat tac cga tgt gac ttc aga act act   528
Leu Leu Leu Lys Gly Asp Val His Tyr Arg Cys Asp Phe Arg Thr Thr
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tac aaa tct agg cag gag ggt gtc aag ttg cca gga tat cac ttt gtc   576
Tyr Lys Ser Arg Gln Glu Gly Val Lys Leu Pro Gly Tyr His Phe Val
            180                 185                 190
gat cac tgc atc agc ata ttg agg cat gac aaa gac tac aac gag gtt   624
Asp His Cys Ile Ser Ile Leu Arg His Asp Lys Asp Tyr Asn Glu Val
        195                 200                 205
aag ctg tat gag cat gct gtt gcc cat tct gga ttg ccg gac aac gtc   672
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Lys Leu Tyr Glu His Ala Val Ala His Ser Gly Leu Pro Asp Asn Val

210 215 220

aag taa 678

Lys

225

[Brief Description of the Drawings]

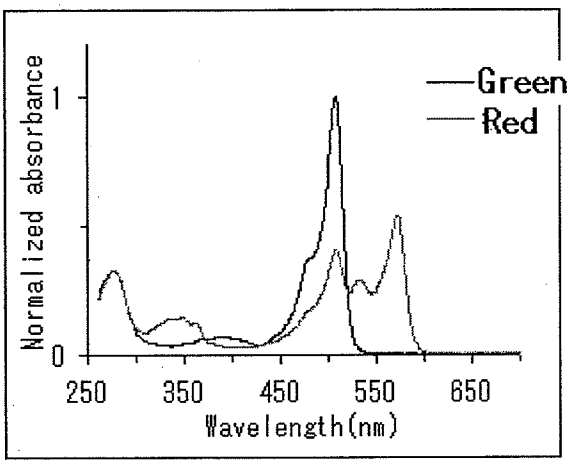
[Fig.1] Figure 1 is the absorption spectrum of the fluorescent protein of the present invention.

[Fig.2] Figure 2 is the fluorescence spectrum of the fluorescent protein of the present invention.

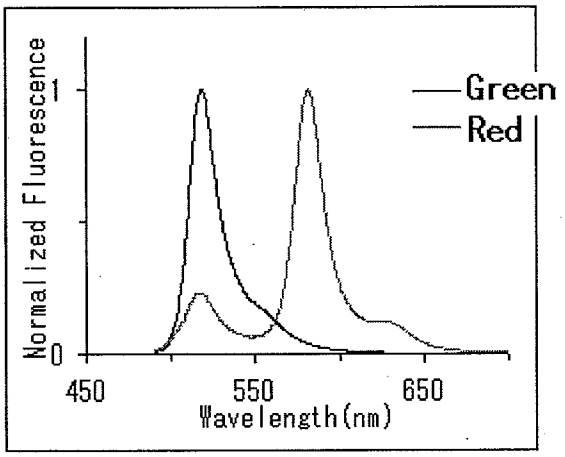
[Fig.3] Figure 3 shows the results obtained by exciting at 470 nm, HeLa cells into which the gene of the fluorescent protein of the present invention has been introduced, and measuring them with the fluorescence at 510 nm.

[Name of Document] Drawing

[Fig.1]



[Fig. 2]



[Fig. 3]

[Name of Document] ABSTRACT

[Abstract]

[Object] To provide a fluorescent protein having a novel primary structure, which is derived from Cnidaria, and particularly from *Trachyphyllia geoffroyi* belonging to Scleractinia.

[Means for Solution] A fluorescent protein derived from *Trachyphyllia geoffroyi*, which has the following properties:

(1) the color is changed from green to red by irradiation of ultraviolet ray; the excitation maximum wavelength is 508 nm (green) and 572 nm (red); and the fluorescence maximum wavelength is 518 nm (green) and 581 nm (red);

(2) the molar absorption coefficient (green) at 508 nm is 98800 $M^{-1}cm^{-1}$; and the molar absorption coefficient (red) at 572 nm is 60400 $M^{-1}cm^{-1}$;

(3) the quantum yield is 0.80 (green) and 0.33 (red); and

(4) pKa regarding the pH sensitivity of the green and red are both 5.7.

[Selected Drawing] None